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13. ABSTRACT *(Maximum 200 Words)*

The goal of this project is to identify proteins that bind to the N-terminal region of the human androgen receptor (hAR). This region is characterized by a series of CAG repeats that is variable in length in different individuals. The scope of this research is the following: 1) to identify N-terminal binding proteins using a yeast two-hybrid system, 2) to confirm the interaction of the candidate proteins with hAR in mammalian cells, 3) to investigate differential binding of these proteins to hAR with varied numbers of CAG repeats, and 4) to examine resultant levels of gene transcription by the different length hAR molecules. Our hypothesis is that unique proteins bind preferentially to hAR proteins with longer CAG repeats, negatively regulating their transcription. Bait plasmids were constructed for the yeast two-hybrid system which contain only the DNA fragment corresponding to the N-terminal 90 amino acids plus Gln repeats of two different lengths (27 and 17). Using the longer sequence as bait, a prostate cDNA library was screened and four positive clones were identified. It is our anticipation that this research will lead to new approaches to abrogate the activity of the androgen receptor in patients with prostate cancer.

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Paul H. Gamlock 10/13/95

PI - Signature Date

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5. INTRODUCTION

This specific goal of this project is to identify proteins that bind to the N-terminus of the human androgen receptor (hAR) including the region encoded by the CAG repeat sequence. The length of the CAG repeat sequence inversely correlates with the aggressiveness of prostate cancer in different ethnic groups, and somatic shortening of the CAG repeat length has been seen in the tumor cells compared to normal constitutional DNA from some prostate cancer patients. This has led to our hypothesis that there will be a protein(s) that binds differentially to the hAR proteins containing different numbers of glutamine (Gln) residues encoded by the CAG repeat. The identification of a protein that down-regulates hAR activity through the Gln repeats may lead to more effective approaches to complete androgen blockade than the currently used approaches that target the carboxy-terminal hormone-binding domain of the receptor. This may then lead to improved therapy for prostate cancer patients. The scope of the research is the following: 1) to identify N-terminal binding proteins using a yeast two-hybrid system, 2) to confirm the interaction of the candidate proteins with the androgen receptor in mammalian cells using a mammalian two-hybrid system, 3) to investigate differential binding of these proteins to fragments of hAR with varied numbers of glutamine residues, and 4) to examine resultant levels of gene transcription activation by the different Gln length hAR molecules.

6. BODY

Due to the goal to isolate unique proteins with binding specificity to a small N-terminal region including the Gln repeat region, we chose to use as bait the region of hAR upstream of that where the transcription factor TFIIF had been shown to bind (**1**). This necessitated the cloning of the region from known hAR variants to obtain wild-type sequence clones of the region with different CAG repeat lengths. RT-PCR was used to amplify the target sequences for cloning the DNA encoding the N-terminus of the receptor. To identify androgen receptors with long and short glutamine repeats RNAs from several prostate cancer cell lines were examined.

PCR cloning of the N-terminus of androgen receptor and construction of the bait plasmids.

To construct bait plasmids for yeast two-hybrid screening, we cloned the DNA fragment encoding the N-terminal 90 amino acids plus the Gln repeat sequence of hAR (**Figure 1, Appendix**). The yeast MACTHMAKER two-hybrid system was purchased from Clontech.

The prostate cancer cell lines which contain the hAR were used to obtain the N-terminus of hAR with longer (> 25 x CAG) and shorter (< 18 x CAG) repeats. Total RNA was prepared from LNCaP cells and PC3 cells transfected with hAR and RT-PCR was performed with primers designed to create the cloning sites. Agarose gel

electrophoresis analysis of the PCR products showed that the DNA fragments generated by the RNA from LNCaP cells were slightly larger than that from PC3 cells (**Figure 2, Appendix**). The PCR products were subcloned into the two-hybrid vector pAS2-1, which created the fusion product of the Gal4 DNA binding domain and the N-terminus hAR region. Subsequent DNA sequencing revealed that the N-terminus of hAR from LNCaP and PC3/hAR cells contained 26 and 16 CAG repeats, respectively. The yeast strain containing reporter genes, beta-galactosidase and *His3* genes, was transformed with these bait plasmids individually. **Figure 3 (Appendix)** shows X-gal assays of those yeast transformants. The yeast strains containing pAS2-1 with 26 CAG repeats (L1-1 and L1-2), the strain pAS2-1 with 16 CAG repeats (S7-1 and S7-2) and the beta-galactosidase positive strains (P1 and P2) were grown on plates containing synthetic glucose medium lacking tryptophane (SD-Trp Medium: Synthetic yeast medium with 2% glucose lacking tryptophane). The plate was subjected to the filter lifting X-gal assay. This assay confirmed that these bait plasmids by themselves did not activate the beta-galactosidase reporter gene **(Figure 3, Appendix)**.

Task 1: To identify proteins encoded by prostate-derived cDNAs that bind the N-terminus of the androgen receptor with 25 glutamine repeats using the yeast two-hybrid system.

Amplification of the cDNA library

A pACT2 cDNA library (human prostate) in *E.coli* was purchased from Clontech. The library was amplified by culturing the bacteria on the solid bacteria medium, and cDNA library plasmids were purified by CsCl gradient centrifugation.

Yeast two-hybrid screening

1) Strategy of the yeast transformation:

Our hypothesis is that unique proteins bind preferentially to the longer glutamine (CAG) repeats and negatively regulate the transcription activity of the androgen receptor. Therefore to screen a prostate library, we used the host yeast strain which carries a bait plasmid with 26 CAG repeats, which is designated as pAS2-1L1.

2) Problems with the yeast transformation.

To set up the screening we faced some difficulties. First, the yeast transformation protocol provided by Clontech did not yield an efficient transformation frequency and was not sufficient to completely screen the library (< $1x10^2$ cfu/mg DNA). To obtain sufficient numbers of transformants to cover the number of independent clones in the library ($3.5x10^6$), the transformation frequency needs to be > $1x10^4$ cfu/mg DNA. This required additional work to establish the transformation protocol. We tried several conditions for culturing the yeast cells, as well as different transformation procedures. With the protocol described below we obtained a transformation frequency of approximately $1x10^4$ cfu/mg

DNA. Transformation with the cDNA library plasmids was performed twice in order to obtain a sufficient number of transformants (total 6.6 x 10^6).

Transformation A = $7.2x10^3$ cfu/mg

Transformation B = $1.9x10^4$ cfu/mg

3) Description of the developed yeast transformation protocol for the two-hybrid screening.

-The host yeast CG1954/pAS2-1L1 was cultured in 100ml of the synthetic minimum medium lacking tryptophane (SD–trp) overnight at 30°C.

-These yeast cells were transferred to 1000ml of fresh SD-trp medium and cultured overnight at 30°C.

- Yeast cells were collected, reinoculated into 500ml of YPD medium and cultured until the A_{660} reached approximately 1.0.

- Refreshed yeast cells were collected by centrifugation and resuspended in 50 ml of One-Step Transformation buffer, which consists of 0.2N LiAc, 40% PEG, 100mM DTT.

-2ml of the cell suspension was aliquoted into 25 tubes and 1mg of salmon sperm DNA (ssDNA) and 10mg of pACT2cDNA library plasmid were added to the each tube.

- Cells were incubated for 30min at 45°C.

- 400ml of the cell suspension was plated onto each of the 100 plates (14cm diameter) containing SD-trp-leu-his medium.

- To determine the transformation efficiency, transformed yeast cells were diluted and plated onto SD-trp-leu plates.

- All the plates were incubated for 7-14 days at 30°C.

4) Problems with screening of the library.

The *His*⁻ phenotype of the host yeast strain CG1945, which is a prime selection marker for the two-hybrid interaction is leaky, which results in the growth of false positive clones on the selection medium. To suppress this growth, the histidine analogue 3-aminotriazole (3-AT) can be added to the selection medium. However, this treatment has the possibly of eliminating positive clones, which have a weak interaction between the bait and the binding proteins expressed by library cDNAs. We decided not to add 3-AT into the selection medium. Almost all of the transformants which grew to a 2mm diameter or larger were tested for beta-galactosidase by X-gal assay. (The transformants which stay smaller than 2mm in diameter are false positives according to the company's instruction manual.) In the case of the selection plates, which contain a large number of transformants of greater than a 2mm diameter, whole plates were subjected to the X-gal assay. Colonies staining blue indicate the positive clones containing a cDNA that encodes a protein that binds with the N-terminus of hAR.

Identification of proteins expressed in the prostate that interact with the N-terminus of the human androgen receptor.

A summary of the the yeast two-hybrid screening is shown in **Table 1 (Appendix)**. The four positive clones identified were re-streaked and cultured on the SD-trp-leu-his plates and the plate was again subjected to the X-gal assay **(Figure 4, Appendix)**. Again these clones showed growth in the abscence of histidine and the blue staining indicating a positive clone.

Immunoprecipitation of the human androgen receptor to identify interacting proteins.

We have begun establishing immunoprecipitation of the human androgen receptor with the goal of identifying interacting proteins that co-precipitate with hAR. We have obtained antibodies that recognize hAR and have begun to examine their utility for the immunoprecipitations and Western blot detection of the receptor. This approach will be used to confirm interactions of the candidate hAR-binding proteins with the androgen receptor in the mammalian two-hybrid system experiments to be initiated in Year 2.

7. KEY RESEARCH ACCOMPLISHMENTS

We have isolated four prostate-derived cDNA clones through multiple rounds of screening that are candidates for encoding proteins that interact by binding with the N-terminal region encoding 27 Gln (26 CAG plus one CAA) repeats of the human androgen receptor. These clones will now be characterized by DNA sequencing of the prostate-derived cDNA insert to identify the specific protein encoded by the cDNA.

8. REPORTABLE OUTCOMES

Expression plasmids containing the hAR N-terminal region with either 26 CAG repeats or 16 CAG repeats were created and confirmed by DNA sequencing.

9. CONCLUSIONS

We have isolated four yeast clones which showed the activation of the His3 and β-galactosidase genes in the yeast two-hybrid system. Since we used the bait construct which contains only the DNA fragment corresponding to the N-terminal 90 amino acids plus the Gln repeat of hAR, the proteins expressed from these candidate cDNA clones are expected to interact directly with this portion of the receptor. It is now important to identify these cDNAs and confirm the interaction with the receptor in a mammalian system. Recently, a coactivator of hAR was identified which binds differentially to different lengths of glutamine repeats (1, 25 and 49 repeats) with the highest affinity to the receptor containing one glutamine (**2**). It is possible that one of the candidates cDNA may encode this coactivator. However, our expectation is to isolate proteins which bind to the longer repeats (27 Gln) with a higher affinity and shorter repeats (17 Gln) with weaker

affinity. Based on our hypothesis, we expect that these hAR binding proteins will down-regulate the transactivating activity of the receptor. If these results are obtained, this will lead to new approaches to abrogate the activity of the androgen receptor in patients with prostate cancer.

10. REFERENCES

1. I.J. McEwan, J. Gustafsson, *Proc. Natl. Acad. Sci. USA* **94**, 8485 (1999).
2. P.-W. Hisao, *et al.*, *J. Biol. Chem.* **274**, 20229 (1999).

APPENDIX

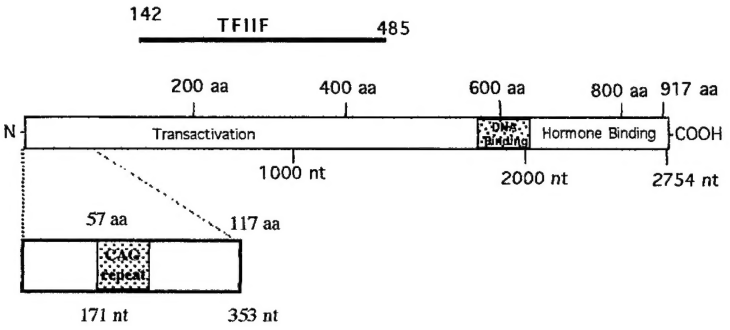

Fig. 1 Schematic presentation of hAR.

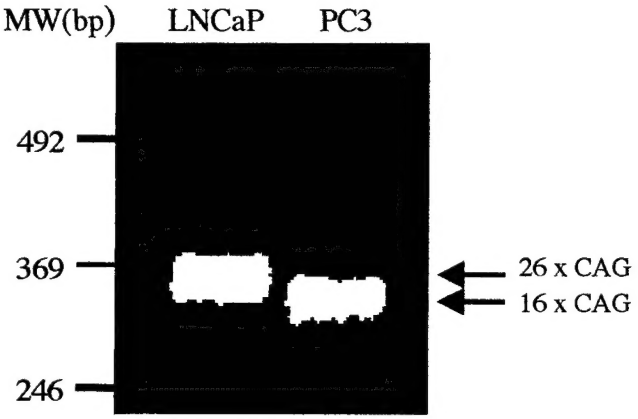

Fig. 2 .Agarose *gel electrophoresis of the PCR products.*

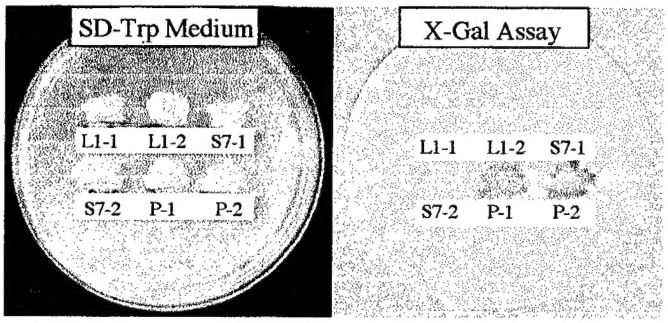

Fig.3 X-gal assay of the yeast cells containing the two-hybrid plasmids with the DNA fragments, which encode N-terminus of hAR. SD-Trp Medium: Synthetic yeast medium with 2% glucose lacking tryptophane. L1-1and L1-2: yeast strains containing pAS2-1 with 26 CAG repeats. S7-1 and S7-2: yeast strains containing pAS2-1 with 16 CAG repeats. P1 and P2: β-galactosidase positive yeast strains.

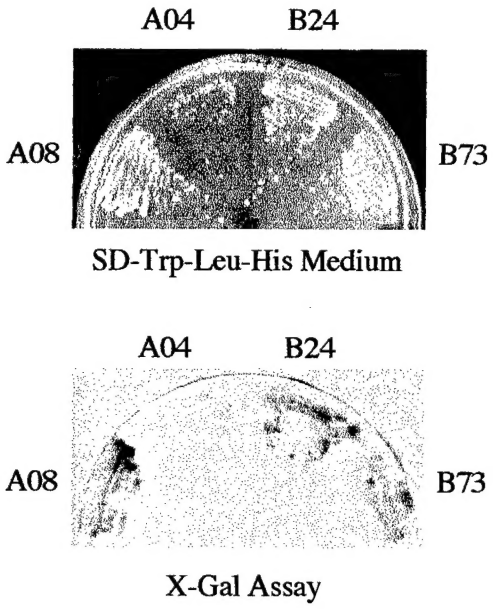

Fig.4 His+ growth and X-gal assay of the positive clones.

Table 1 Summary of the screening

Transformation	Number of transformants screened	Number of His+ transformants isolated	Number of β-galactosidase positive transformants
1	1.8×10^6	24	2
2	4.8×10^6	73	2
total	6.6×10^6	97	4